A Food-Grade Cloning System for Industrial Strains of *Lactococcus lactis*

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We have previously reported the construction of a food-grade cloning vector for *Lactococcus* using the ochre suppressor, *supB*, as the selective marker. This vector, pFG1, causes only a slight growth inhibition in the laboratory strain MG1363 but is unstable in the industrial strains tested. As *supB* suppresses both amber and ochre stop codons, which are present in 82% of all known lactococcal genes, this undesirable finding may result from the accumulation of elongated mistranslated polypeptides. Here, we report the development of a new food-grade cloning vector, pFG200, which is suitable for overexpressing a variety of genes in industrial strains of *Lactococcus lactis*. The vector uses an amber suppressor, *supD*, as selectable marker and consists entirely of *Lactococcus* DNA, with the exception of a small polylinker region. Using suppressible pyrimidine auxotrophs, selection and maintenance are efficient in any pyrimidine-free medium including milk. Importantly, the presence of this vector in a variety of industrial strains has no significant effect on the growth rate or the rate of acidification in milk, making this an ideal system for food-grade modification of industrially relevant *L. lactis* strains. The usefulness of this system is demonstrated by overexpressing the *pepN* gene in a number of industrial backgrounds.

Lactococcus lactis is an important organism for the dairy industry, where it is used for the production of many cheese varieties as well as cultured milk products such as buttermilk. *Lactococcus* contributes to the successful manufacture of these products through the production of lactic acid and a variety of flavor components. While excellent industrial strains do exist, there is a constant desire in the dairy industry for strains with improved properties. One useful method for strain improvement is genetic engineering, provided, of course, that the genetic modifications are food grade (10). A number of characteristics, such as flavor and acid production, bacteriophage resistance, proteolytic activity, and the autolytic properties of strains, can be improved by overexpressing the relevant genes (3, 4, 5, 10). A simple method for overexpressing a gene is to transfer it to a multicopy cloning vector.

We have previously described the construction of a food-grade cloning vector for *Lactococcus* (5). This vector, pFG1, uses an ochre suppressor gene, *supB*, as the selectable marker. It exists in five to nine copies in the cell and has allowed the overexpression of several *Lactococcus* genes in strains derived from the laboratory strain MG1363. The use of pFG1 in industrial strains of *Lactococcus* has not been successful, however, due to a reduced acidification rate in strains bearing pFG1 derivatives (unpublished results). Whereas in MG1363 derivatives pFG1 causes a slight growth inhibition, in industrial strains a severe growth inhibition is seen and the vector is very unstable. Since an ochre suppressor suppresses both amber and ochre codons and 82% of *Lactococcus* genes terminate in either amber or ochre stop codons (2, 5), this growth inhibition is probably caused by the accumulation of elongated, mistranslated polypeptides. Based on these observations, we have developed a new food-grade cloning vector, pFG200, using an amber suppressor, *supD*, as a selectable marker. *supD* encodes

an altered tRNA^{Ser} (5) and suppresses only amber codons, which are present at the ends of approximately 10% of *Lactococcus* genes (2).

A powerful selection system has been constructed in parallel with the vector. By introducing an amber codon into the *pyrF* gene, we generated pyrimidine auxotrophs to use as host strains for pFG200. This marker allows the selection and maintenance of pFG200 in pyrimidine-free medium. The pyrimidine content of milk is insufficient for growth of these pyrimidine auxotrophs, so the selection also works in milk.

In this paper, we demonstrate the potential of this system by illustrating that pFG200 is stably maintained in the host cells in media including milk, has virtually no impact on growth and acidification rates, and, finally, enables a cloned gene such as *pepN* (15) to be overexpressed.

MATERIALS AND METHODS

Bacterial strains and media. Strains of *L. lactis* and *Escherichia coli* are listed in Table 1. *L. lactis* was grown in M17 (16) or DN minimal medium (5). Erythromycin was used at 1 µg/ml when required. *E. coli* was grown in Luria-Bertani medium, supplemented with ampicillin to 50 µg/ml when required. *pyrF*(Am) mutants were cultivated in M17 or DN minimal medium, both with 20 µg of uracil per ml and 40 µg of thymidine per ml added. Selection and maintenance of plasmids bearing *supD* in *pyrF*(Am) strains were in DN minimal medium without added pyrimidines. Growth was at 30°C and was measured by monitoring the increase in optical density at 600 nm. Experiments to determine the plasmid stability and acidification rate were carried out in reconstituted skim milk (RSM) and pasteurized whole milk, respectively.

Competent cells and electroporation. Preparation of competent *L. lactis* cells in rich medium containing glycine and electroporation were as described previously (8). For MG1363, CHCC3052, CHCC270, and derivatives, 1% (wt/vol) glycine was used, while for CHCC377 and derivatives, 1.5% (wt/vol) glycine was used. Pyrimidine auxotrophs were grown in medium containing 20 µg of uracil per ml and 20 µg of thymidine per ml to reduce the frequency of revertants. Transformation of *E. coli* was done essentially as described by Sambrook et al. (14).

DNA purification and manipulation. Small-scale plasmid preparations from *E. coli* were by alkaline lysis, and large-scale preparations were with the Qiagen system. Plasmid DNA and chromosomal DNA were isolated from *L. lactis* as described previously (5) with the modification that 50 µl of 5 M NaCl was added before the phenol extraction when plasmids were purified. DNA sequencing was by cycle sequencing as recommended by Perkin-Elmer. The resulting products

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TABLE 1. Bacterial strains

Strain	Description	Source (reference)
L. lactis		
MG1363	Plasmid-free derivative of NCDO 712	Gasson (6)
FA4-1-1	Pyrimidine auxotroph of MG1363 (CAA$_{154}$ → TAG)	This study
CHCC4210	Pyrimidine auxotroph of MG1363 (TCT$_{127}$ → TAG)	This study
CHCC377	Industrial strain of *L. lactis* subsp. *lactis* (major component of a highly successful commercial culture)	CHCC[a]
CHCC4146	Pyrimidine auxotroph of CHCC377 (TCT$_{127}$ → TAG)	This study
CHCC4223	CHCC4146(pFG200)	This study
CHCC3052	Industrial strain of *L. lactis* subsp. *lactis*	CHCC
CHCC4205	Pyrimidine auxotroph of CHCC4205 (TCT$_{127}$ → TAG)	This study
CHCC270	Industrial strain of *L. lactis* subsp. *lactis*	CHCC
CHCC4424	Pyrimidine auxotroph of CHCC4205 (TCT$_{127}$ → TAG)	This study
E. coli DH5α	*deoR endA1 gyrA96 hsdR17* (r_K^- m_K^+) *relA1 recA1 supE44 thi-1* Δ(*lacZYA-argF*)U169/φ80dlacΔ(*lacZ*)M15	Hanahan (7)

[a] CHCC, Chr. Hansen Culture Collection.

were purified on Bio-Rad Micro-Bio-Spin-Chromatography columns before being run on an ABI PRISM 310 Genetic Analyzer. The primers used for sequencing and PCRs are listed in Table 2.

Sequencing of the *pyrF* gene. The nucleotide sequence of the CHCC377 *pyrF* gene was obtained by first producing a 550-bp internal *pyrF* fragment via PCR using two degenerate primers (pyrF3 and pyrF4), which were designed based on amino acid sequence comparison of the *pyrF* proteins of a number of microorganisms. The fragment was sequenced with the same primers. New primers (pyrF5 and pyrF6) were obtained based on this sequence and used for inverse PCR with Sau3A1-digested DNA, giving additional sequence data. The sequence of the amino-terminal end of *pyrF* was completed using a degenerate primer designed from the sequence of *pyrD* (pyrD-dgen), which is immediately upstream of *pyrF*, and two additional primers (pyrF7 and pyrF8). The carboxy-terminal end of *pyrF* was sequenced using a degenerate primer (llagidB2) designed from the sequence of *gidB*, which is immediately downstream of *pyrF*, and pyrF6.

Plasmid constructions. The construction of pFG200 is described in detail in Results. The *pepN* gene of *L. lactis* strain Wg2 was subcloned from pSTO3 (15) as a 3.5-kbp BamHI-SacI fragment, including the promoter region, into the corresponding sites in the pFG200 polylinker to generate pFG202. The promoterless *lacLM* genes were subcloned from pAK80 (9) as a HindIII-SalI fragment either into HindIII-SalI-digested pFG200, generating pFG209, or into HindIII-XhoI-digested pFG200, generating pFG208. These two plasmids represent the two possible orientations of *lacLM* in pFG200.

Plasmid copy number determination. Total genomic DNAs isolated from fresh saturated cultures of CHCC4210 and CHCC4210/pFG200 grown in DN medium were digested with HindIII. After separation by agarose gel electrophoresis, the DNA was transferred to a GeneScreen Plus nylon filter (E.I. du Pont de Nemours). A 298-bp PCR fragment containing *supD*, generated using the primers amber-3 and amber-6 with pAK93 DNA as the template, was used as a hybridization probe. The probe was labeled with [α-^{32}P]dATP, using the Mega-

prime labeling kit (Amersham), for 3 h at 37°C. Hybridization was carried out at 60°C in 6× Denhardt's solution and the filter was washed three times, as described by Sambrook et al. (14). The level of ^{32}P in each band was quantified with an Instant-Imager (Packard Instrument Co.). The copy number of the vector was calculated as the ratio of counts per minute in the vector band to counts per minute in the chromosomal band (average of three determinations).

Enzyme assays. Cultures were grown to saturation in DN medium. After sonication, the cell extracts were used to determine either PepN activity (using L-lysine-*p*-nitroanilide [15]) or β-galactosidase activity (using *o*-nitrophenyl-β-D-galactoside [9]). One unit of lysine aminopeptidase will hydrolyze 1 nmol of substrate min^{-1}. β-Galactosidase activity, in Miller units, was calculated as described previously (9). Protein determinations were done with a Protein Assay Kit I (Bio-Rad).

Acidification rate. Evaluation of acidification rates was by measuring the pH following inoculation of cultures as single strains into pasteurized whole milk. The temperature was controlled by an automatic temperature controller, generating a modified cheddar cheese temperature profile. The milk had been stored overnight at 4 to 7°C in bottles with loose lids, in order to ensure equal oxygen levels in all bottles. The pH was measured semicontinuously throughout 16 h of incubation with an AAC data-logger from Intab A/B. Acidification curves were generated by the Easyview software package, version 3.2.0.4.

Nucleotide sequence accession number. The DNA sequence of the *pyrF* gene appears in the EMBL and GenBank nucleotide sequence data library under accession number AF174425.

RESULTS

Sequencing and cloning of *pyrF* genes from industrial strains. The most precise way to obtain a *pyrF*(Am) mutant is to construct the mutation in vitro via PCR (see below) and then

TABLE 2. Primers

| Primer	Sequence
For MG1363	
Fam3	5'-AACAGCCTAGGCCGGACTTGATGG-3'
Fam4	5'-GTCCGGCCTAGGCTGTTTTTTGTGCG-3'
pyrF1	5'-GCAGATCTAAGCTTGATTCAAGAAGTAAAAGAAGGC-3'
pyrF2	5'-ATAGATCTACTCGATGCCAAGAATGGACCGC-3'
For CHCC377	
pyrF3	5'-AAAGGCCTGTNATNGCNCTNGAYTTYCC-3'
pyrF4	5'-TGGACGAATTCCNGGNGT-3'
pyrF5	5'-CATAGTAAACGACTTGGGG-3'
pyrF6	5'-TACGCACAAAAAACCGCT-3'
pyrF7	5'-GGTCGCCTTTACTTGCACC-3'
pyrF8	5'-GATTATATTGTTGTCGGCCG-3'
pyrD-degn	5'-GCTCTAGAGCMWATYGWWATDGGN-3'
llagidB2	5'-GGTNGARTGGAAYGARAARATH AAY-3'
Fam5	5'-CCTCAACCTAGGAGAAAATTATGC-3'
Fam6	5'-TCTCCTAGGTTGAGGTTAATTGTG-3'
pyrD/BglII	5'-ATAGATCTGCTTAGAAAACTTG-3'
pyrF11/BglII	5'-ATAGATCTGCATGTAAGCAAAAACC-3'
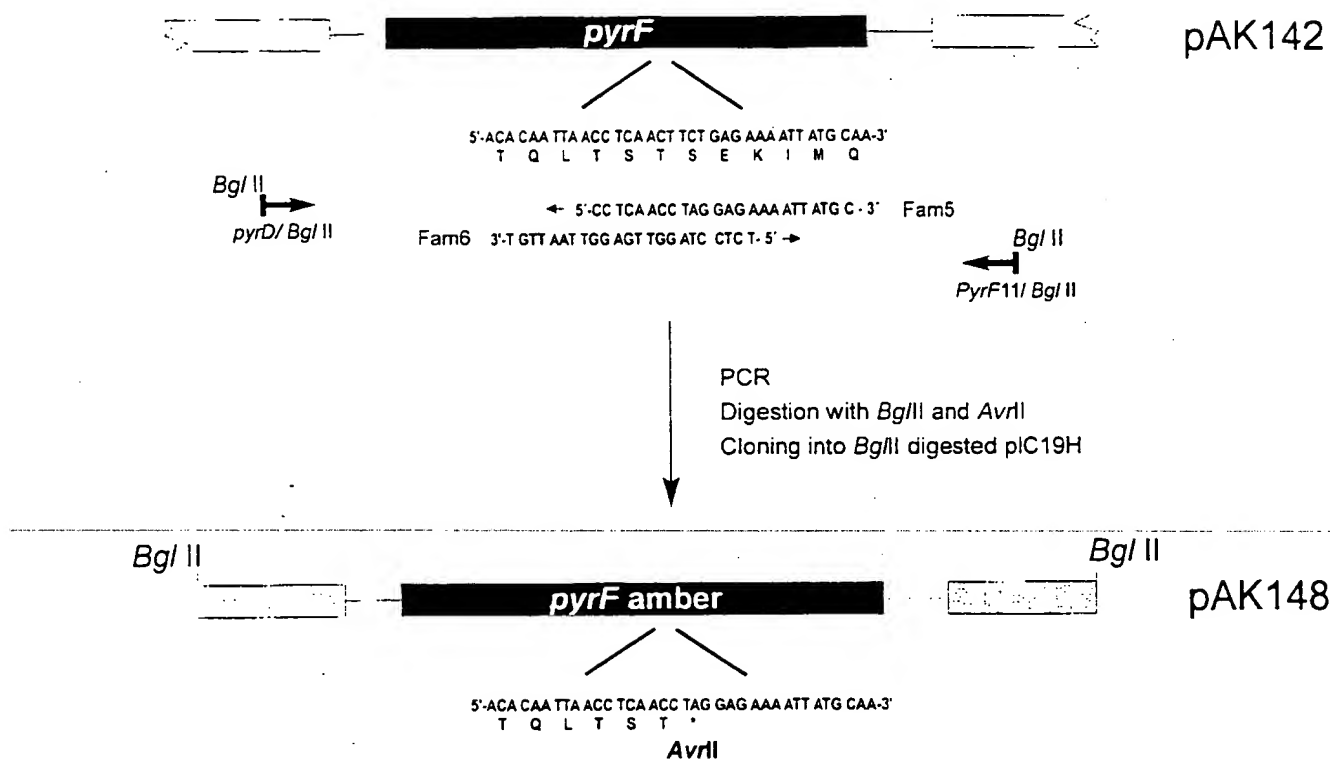
FIG. 1. Construction of a *pyrF*(Am) mutation. The given example illustrates the construction made for the CHCC377 *pyrF* gene. Plasmid pAK142, carrying the entire *pyrF* gene, is used as template DNA in a PCR. The two overlapping primers, Fam5 and Fam6, containing the substitutions to generate an amber codon within an *Avr*I site, were combined with a primer upstream of *pyrF* (PyrD/BglII) and a primer downstream of *pyrF* (PyrF11/BglII). The two resulting PCR products were digested with *Avr*II and *Bgl*II, mixed, and cloned. The resulting plasmid, pAK148, carries the desired amber mutation in *pyrF*.

to introduce the mutation into the chromosome by homologous recombination (10). Attempts to use MG1363 *pyrF*(Am) DNA to introduce a *pyrF*(Am) mutation into some industrial strains were unsuccessful, presumably due to insufficient homology between the *pyrF* genes of MG1363 and the industrial strains used. We determined the DNA sequence of the CHCC377 *pyrF* gene and found it to be 86% identical to that of MG1363 (1). Sequencing of *pyrF* genes from other industrial strains revealed that *L. lactis* subsp. *lactis* strains had sequences nearly identical to that of CHCC377 and that *L. lactis* subsp. *cremoris* strains had sequences nearly identical to that of MG1363.

Introduction of an amber mutation into a cloned *pyrF* gene. The procedure for introduction of the mutation into the *pyrF* gene is the same for each host strain. The DNA sequence is searched for a serine codon that can be changed to an amber codon and which is flanked by sequences that allow the introduction of a restriction site without affecting the amino acids encoded by the flanking sequences. PCR is then used to introduce the desired alterations.

For CHCC377, the entire *pyrF* gene was cloned as a 1.1-kb PCR fragment in pIC19H (12) to produce pAK142 (Fig. 1). Two overlapping PCR primers, Fam5 and Fam6, contain the substitutions that generate an amber codon and an *Avr*II restriction site (Fig. 1). When Fam5 is used in combination with a primer upstream of *pyrF* (PyrD/BglII) and Fam6 is used with a downstream primer (PyrF11/BglII), with pAK142 as template DNA, two PCR products are generated. These were

digested with *Avr*II and *Bgl*II, mixed, and cloned into *Bgl*II-digested pIC19H. The resulting clone, pAK148, contains the *pyrF* gene with the desired amber mutation. Suppression of the amber mutation by *supD* will introduce a serine residue resulting in an enzyme identical to that of the parent strain.

A similar approach was used to construct amber mutations in the *pyrF* genes of other strains. The host strain, FA4-1-1, was derived from MG1363 but constructed slightly differently to serve as a host for both pFG1 and pFG200 (see below). Using the approach described above and in Fig. 1 with other primers (Table 2), an amber stop codon was introduced at glutamine codon 154 in the *pyrF* gene. Suppression by *supD* will insert serine, while suppression by *supB*, contained on pFG1, causes insertion of glutamine in the synthesized *pyrF* enzyme.

Introduction of the amber mutation into the chromosome. The DNA fragments carrying the *pyrF* amber mutations of MG1363, CHCC377, and CHCC270 were subcloned into pG⁺Host9 (11) to produce the plasmids pAK133, pAK149, and pRL202, respectively. The resulting pG⁺Host-derived vectors were then used to integrate the amber mutations into the corresponding chromosomal *pyrF* genes. Strains with the desired *pyrF*(Am) allele are found by screening survivors isolated on M17 plates at 30°C for their pyrimidine requirement (10). The *pyrF*(Am) derivatives of MG1363 were named CHCC4210 (serine to amber) and FA4-1-1 (glutamine to amber). The *pyrF*(Am) derivatives of CHCC377, CHCC3052, and CHCC270 were named CHCC4146, CHCC4205, and CHCC4424, respectively.

EcoRV BglII SacI
EcoRI   XbaI   XhoI   NruI HindIII PstI   SalI BamHI   EcoRI
GAATTCATCGATATCTAGATCTCGAGCTCGCGAAAGCTTGGCTGCAGGTCGACGGATCCCCGGGAATTC
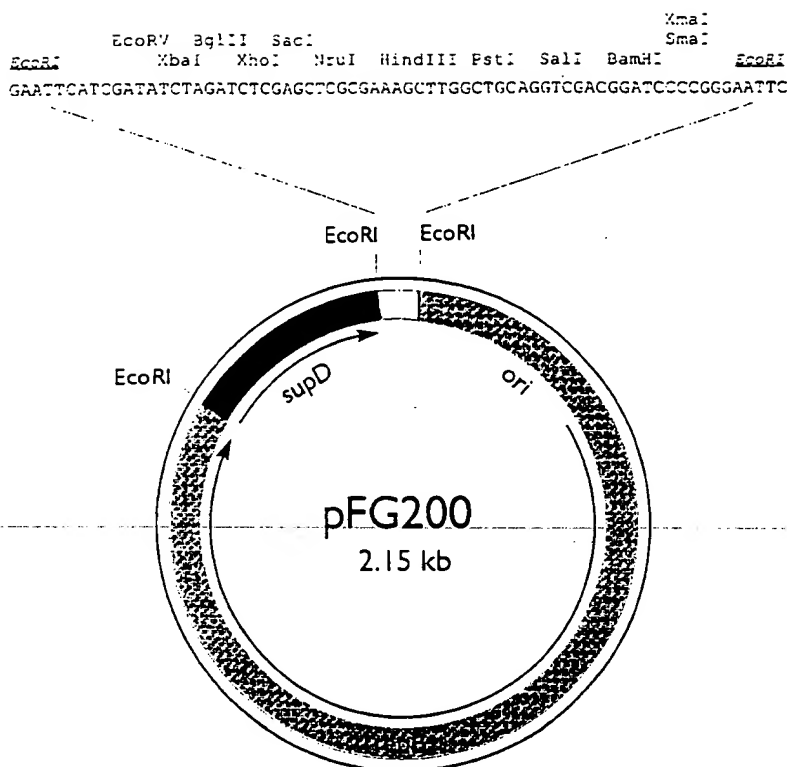
Xmai
Smai

EcoRI   EcoRI

FIG. 2. Map of food-grade vector pFG200. The components used for the construction are described in the text. Arrows indicate the direction of transcription of repB and supD. Except for EcoRI, only those restriction sites that are unique to the polylinker are shown.

Construction of the food-grade vector pFG200. The construction of pFG200 was based on the strategy previously described for pFG1 (5). Plasmid pKR41 contains the *L. lactis* subsp. *lactis* biovar diacetylactis citrate plasmid replicon cloned in pIC19H (13). PCR was performed on pAK89.1 using primers amber-2 and amber-3 to produce a fragment with *EcoRI* ends that contains the *supD* gene (5). After digestion with *EcoRI*, the 347-bp PCR product was cloned into the *EcoRI* site of pIC19H to generate pAK93. Plasmids pKR41 and pAK93 were both digested with *EcoRI*, mixed, and ligated. The resulting ligation mixture was electroporated into FA4-1-1, selecting Pyr⁻ transformants. Among the plasmids obtained, approximately half contained the citrate plasmid replicon, *supD*, and pIC19H. One of these. pMPJ103. was used to construct pFG200. To remove all of pIC19H except the polylinker, pMPJ103 was digested with *Hind*III, ligated, and transformed into FA4-1-1. The resulting Pyr⁻ transformants contained a 2.15-kb plasmid. which was named pFG200 (Fig. 2).

Electroporation of pFG200 into FA4-1-1 and CHCC4210 is very efficient and typically results in >10⁶ transformants per μg of DNA. The copy number of pFG200 was found to be 6 to 11. The efficiency of transformation of CHCC4146 and derivatives of other industrial strains is significantly lower due to the difficulty of making good competent cells.

Plasmid stability in milk. A fresh overnight culture of CHCC4223 (CHCC4146/pFG200) in minimal medium was subcultured 1:100 in RSM and incubated overnight. The resulting culture was plated on M17 plates and also subcultured 1:100 in RSM. This procedure was repeated for five consecu-

tive days. Each outgrowth was taken to be seven generations. After each outgrowth. 100 single colonies were picked and patched onto minimal plates and minimal plates containing uracil to screen for plasmid-harboring cells. Plasmid preparations were used to ensure that the isolated colonies contained pFG200 and had the expected plasmid profiles. No plasmid loss was observed after 35 generations in milk.

Growth and acidification rates of strains harboring pFG200. In DN minimal medium. MG1363 and CHCC4210/pFG200 grew with identical growth rates ($t_{1/2}$ = 63 min). whereas FA4-1-1/pFG200 grew with a lower growth rate ($t_{1/2}$ = 77 min). The lower growth rate of FA4-1-1/pFG200 is most likely due to a less active PyrF enzyme in that strain resulting from the amino acid substitution at position 154.

The acidification of milk by CHCC377 and CHCC4223 showed the high rate of acidification characteristic for CHCC377 (Fig. 3). This is a significant improvement over the pFG1 system. where the acidification rates for various CHCC377 derivatives were considerably reduced (unpublished results).

Measurement of transcriptional readthrough. To determine if there is any transcriptional readthrough into the polylinker region, promoterless *lacLM* (9) was cloned in both orientations into the polylinker of pFG200 and the β-galactosidase activity was measured. A very low expression of β-galactosidase, corresponding to 5 to 10 Miller units. was detected. with no significant difference between the orientations. revealing little or no transcription into the polylinker.
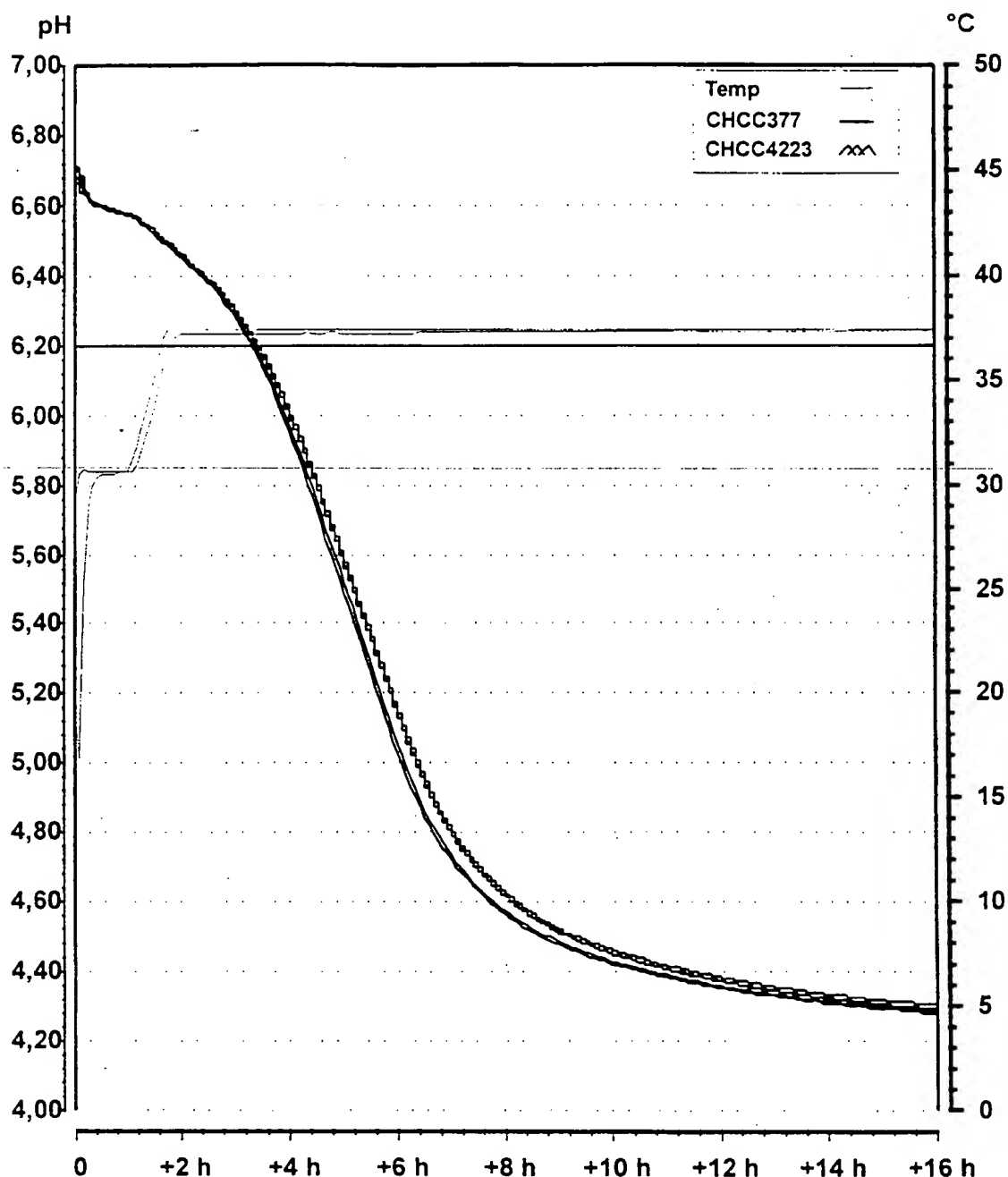
FIG. 3. Acidification curve showing the change in pH during 16 h in whole milk inoculated with strain CHCC377 or strain CHCC4223 (double determinations). The temperature (Temp) profiles for this modified cheddar cheese model system (water bath and bottle) are also shown.

Assay of lysine aminopeptidase activity expressed from pFG202. The *pepN* gene from *L. lactis* strain Wg2 was cloned into pFG200 to generate pFG202. After electroporation into CHCC4210. CHCC4146. CHCC4205. and CHCC4424, the levels of lysine aminopeptidase activities were determined (Table 3). Significant increases in *pepN* activities were seen in all backgrounds.

DISCUSSION

Existing food-grade cloning vectors for *Lactococcus* have been optimized for laboratory strains. Reports of their use in industrial strains are absent, possibly because of a lack of success. For example, pFG1 (5) has been successfully used to overexpress a number of genes in MG1363. but introduction of

TABLE 3. Overexpression of *pepN* by cloning into pFG200

Strain	Lysine aminopeptidase activity (U/mg of protein)	Fold induction
CHCC4210	339	8
CHCC4210/pFG202	2,827	
CHCC4146	89	20
CHCC4146/pFG202	1,822	
CHCC4205	104	23
CHCC4205/pFG202	2,302	
CHCC4424	100	23
CHCC4424/pFG202	2,304	

this vector into industrial strains resulted in acidification rates unsuitable for industrial-scale cheese production (unpublished results).

An improved food-grade cloning system for industrial strains of *L. lactis* has been developed. It is based on a multicopy vector, pFG200 (Fig. 2), constructed without the use of DNA from species other than *Lactococcus*. It contains the replicon fragment of pCT1138 (13), the amber suppressor *supD* from *Lactococcus* strain NJ1 (5) as the selectable marker, and a versatile polylinker. To select for the plasmid, we use the suppression of pyrimidine auxotrophs constructed by introduction of an amber codon in the chromosomal *pyrF* gene (Fig. 1). This system allows selection and maintenance of the vector in milk, which is essentially pyrimidine free. All the advantages of our previously described food-grade cloning vector, pFG1, are also offered by pFG200. These include (i) easy cloning of genes into a versatile polylinker region; (ii) a small and stable multicopy vector which is electroporated into *Lactococcus* with high efficiency; and (iii) a selection system allowing selection and maintenance in milk, which allows genetic modification, cloning, and overexpression of *Lactococcus* DNA in a food-grade manner.

The orientation of a gene inserted in the polylinker of pFG1 affects expression due to a putative promoter downstream of *supB* (unpublished results). Cloning of *lacLM* into pFG200 in both orientations and measurement of β-galactosidase activity showed a very low expression, indicating the absence of significant readthrough from either side of the polylinker.

Full tolerance to multiple copies of *supD* is indicated by several observations. The plasmid is stably maintained in the host cells for more than 35 generations in media including milk. The growth rates of cells with and without pFG200 in both defined media and milk show no differences. Likewise, the presence of pFG200 in important industrial starters has no impact on the acidification rate in milk, an important criterion for the usefulness of these strains in cheesemaking (Fig. 3).

The functionality of the pFG200 system was also tested and demonstrated by cloning the *pepN* gene from strain Wg2 (15) into pFG200 to generate pFG202. The levels of lysine aminopeptidase in the various host strains either with or without pFG202 were determined (Table 3). The endogenous level of lysine aminopeptidase was found to vary from 89 to 339 U/mg of protein. Introduction of pFG200 did not alter these endogenous levels (data not shown). Thus, the level of lysine aminopeptidase encoded by the chromosomal *pepN* gene is strain

dependent (Soren Herskind, personal communication). In all strains, we found a significant increase in lysine aminopeptidase activity when pFG202 is present, which we presume to result from the increased copy number of *pepN*.

Promising results have been obtained in cheese trials and taste tests using MG1363-derived strains containing pFG1 derivatives harboring various aminopeptidase genes or the φvML3 lysin gene (unpublished data). We have also cloned the *pepA*, *pepC*, *pepO*, *pepT*, *pepV*, and *pepX* genes, as well as the φvML3 lysin gene, into pFG200 (unpublished results; Per Strøman, personal communication) and will assess the effect of overexpression of these genes in industrial strains.

The improved food-grade cloning system described here thus allows the overexpression of technologically relevant genes in industrially important strains, providing great promise for future food-grade genetic modifications of *L. lactis*.

ACKNOWLEDGMENTS

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